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## APPENDIX II

[CANCER RESEARCH 61, 4483-4489, June 1, 2001]

Immunophenotyping of Leukemias Using a Cluster of Differentiation Antibody Microarray<sup>1</sup>Larissa Belov, Odetta de la Vega, Cristobal G. dos Remedios, Stephen P. Mulligan, and Richard I. Christopherson<sup>2</sup>

Departments of Biochemistry [L.B., O.d.l.V., R.I.C.] and Anatomy and Histology, Institute for Biomedical Research [C.G.d.R.], University of Sydney, NSW 2006, and Department of Hematology, Concord Hospital, Concord, NSW 2139 [S.P.M.], Australia

## ABSTRACT

Different leukemias express on their plasma membranes particular subsets of the 247 defined cluster of differentiation (CD) antigens, which may resemble those of precursor cells along the lineages of differentiation to mature myeloid and lymphoid leukocytes. The extent of use of CD antigen expression (immunophenotyping) for identification of leukemias has been constrained by the technique used, flow cytometry, which commonly specifies only three CD antigens in any one assay. Currently, leukemias and lymphomas are diagnosed using a combination of morphology, immunophenotype, cytochemistry, and karyotype. We have developed a rapid, simple procedure, which enables concurrent determination of 50 or more CD antigens on leukocytes or leukemia cells in a single analysis using a microarray of antibodies. A suspension of cells is applied to the array, and cells only bind to antibody dots for which they express the corresponding CD antigen. For patients with significantly raised leukocyte counts, the resulting dot pattern then represents the immunophenotype of those cells. For patients at earlier stages of disease, the diagnosis depends on recognition of dot patterns distinct from the background of normal leukocytes. Distinctive and reproducible dot patterns have been obtained for normal peripheral blood leukocytes, chronic lymphocytic leukemia (CLL), hairy cell leukemia, mantle cell lymphoma, acute myeloid leukemia, and T-cell acute lymphoblastic leukemia. The consensus pattern for CD antigen expression found on CLL cells taken from 20 patients in descending order of cells bound was CD44, HLA-DR, CD37, CD19, CD20, CD5, CD52, CD45RA, CD22, CD24, CD45, CD23, CD21, CD71, CD11c, and CD9. The antigens that provided the best discrimination between CLL and normal peripheral blood leukocytes were CD19, CD20, CD21, CD22, CD23, CD24, CD25, and CD37. Results obtained for the expression of 48 CD antigens from the microarray compared well with flow cytometry. The microarray enables extensive immunophenotyping, and the intact cells captured on antibody dots can be further characterized using soluble, fluorescently labeled antibodies.

## INTRODUCTION

Blood cells are derived from stem cells that differentiate and proliferate down the myeloid and lymphoid lineages (1, 2) under the control of cytokines such as colony stimulating factors. Precursor cells from different lineages express different subsets of surface molecules, many of which are now defined by CD<sup>3</sup> antigens. CD antigens associated with the plasma membranes of leukocytes may be molecules involved in a variety of functions (cell-cell interactions, cytokine receptors, cell signaling, ion channels, transporters, enzymes, immunoglobulins, or adhesion molecules; Ref. 2). As cells differentiate along particular lineages, expression of CD antigens changes; e.g., as

myeloid stem cells expressing CD34 differentiate down the granulocyte lineage, they begin to express CD13 and CD33 and down-regulate CD34 (3). Mature neutrophils express CD11b, CD13, and CD15, with the loss of expression of CD33. The expression of CD antigens on leukocytes is currently determined by flow cytometry (4), which is expensive and labor-intensive, requiring 5–20- $\mu$ l quantities of fluorescently labeled antibodies (10–500  $\mu$ g/ml) and allowing concurrent analysis for a limited number of CD antigens, usually three to four.

The main types of leukemias are ALL derived from immature T- or B-lymphocytes, AML from immature myeloid cells, CLL from mature B-lymphocytes, and chronic myeloid leukemia from granulocyte precursors (5). NHL may also enter a leukemic phase with circulating lymphoma cells (5). Accurate diagnosis of hematological malignancies enables selection of the most effective treatment protocol. Current diagnosis of acute leukemias is based on the morphology and cytochemistry of the blast cells according to the WHO classification released recently (6) and the FAB classification used previously (7, 8), usually supplemented with karyotyping and limited immunophenotyping (5). Flow cytometric analysis of leukemias with panels of monoclonal antibodies now provides 98% accuracy for distinguishing acute leukemias of myeloid and lymphoid origin (9) and may differentiate a variety of chronic leukemias and lymphomas (5). Subgrouping of CLL into typical and atypical on the basis of morphology according to WHO/FAB criteria (6, 7) has prognostic significance, and several studies (10) have shown a strong correlation between atypical morphology, trisomy 12, and an aberrant immunophenotype.

In AML, mutations may alter the developmental program resulting in proliferation of cells blocked at a particular stage of differentiation to granulocytes or monocytes. Alternatively, AML could arise from leukemic stem cells that differentiate in an unusual manner (11). Using the WHO/FAB classification system, AML has been divided into multiple subgroups based on: morphology; reaction to peroxidase and Sudan black stains; expression of CD13, CD14, CD33, CD41, CD61 and glycophorin A; types of cytoplasmic granules; Auer rods; vacuoles; chromosomal translocations (8;21 or 15;17); inversion of chromosome 16, 11q23 abnormalities; nonspecific esterase and chloroacetate esterase activities; serum and urinary lysozyme levels; and periodic acid-Schiff staining (6, 12). An experienced hematologist is required to decide which tests should be performed on a particular AML sample. Jennings and Foon (4) reviewed the application of flow cytometry to the diagnosis of leukemias and lymphomas based upon patterns and intensity of antigen expression. They found that limited immunophenotypes did not uniquely define the FAB classification of AML. However, screening for expression of 50–100 antigens could yield consensus patterns corresponding to the existing classes and may facilitate biologically relevant revision of the WHO myeloid neoplasia classification (6). Bain (see Table 2.4 in Ref. 5) showed that the eight different FAB subclasses of AML have different levels of expression of a panel of nine surface antigens. For ALL, immunophenotyping plays a central role in defining clinically relevant subsets. The WHO classification states that ALL should be classified by the pattern of reactivity of cells to a panel of lineage-associated antibodies and, where possible, genetic abnormalities (6).

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<sup>1</sup> This study is dedicated to the memory of Lee Dixon who had M4 acute myeloid leukemia. Supported by Enterix Proprietary Limited (Ryde, NSW, Australia).

<sup>2</sup> To whom requests for reprints should be addressed, at Department of Biochemistry, University of Sydney, NSW 2006, Australia. Phone: 61-2-9351-6031; Fax: 61-2-9351-4726; E-mail: ric@biochem.usyd.edu.au.

<sup>3</sup> The abbreviations used are: CD, cluster of differentiation; ALL, acute lymphocytic leukemia; AML, acute myeloid leukemia; CLL, chronic lymphocytic leukemia; FAB, French-American-British; FACS, fluorescence-activated cell sorter; HCL, hairy cell leukemia; LD Array, a microarray of antibodies against CD antigens named in memory of Lee Dixon; NHL, non-Hodgkin lymphoma; PE, phycoerythrin; GPA, glycophorin A.

## ANTIBODY MICROARRAY FOR LEUKEMIA DIAGNOSIS

Golub *et al.* (13) used gene expression monitoring with DNA microarrays to distinguish between human AML and ALL in bone marrow aspirates from 38 patients. Quantitative levels of expression were obtained for 6817 genes and approximately 1100 genes correlated with the AML-ALL class distinction. Fifty of the genes that showed the closest correlation with the AML-ALL distinction were used to classify new samples with high accuracy. Alizadeh *et al.* (14) used a "Lymphochip" consisting of 17,856 cDNA clones on a microarray to look at gene expression in diffuse large B-cell lymphoma in 96 normal and malignant lymphocyte samples. Two distinct forms of the lymphoma were identified with gene expression patterns indicative of different stages of B-cell differentiation. The use of oligonucleotide arrays described in these papers and others to classify leukemias or lymphomas is empirical and complex to perform, does not interface with current diagnostic criteria (morphology, immunophenotype, cytochemistry, and cytogenetics), and lacks clinical correlation. Furthermore, there is an uncertain relationship between levels of mRNA and protein and any subsequent post-translational modification.

Chang (15) demonstrated specific binding of human peripheral blood mononuclear cells to mouse antihuman HLA-A2 antibody (50 nM) adsorbed to glass coverslips and binding of mouse thymocytes to similarly immobilized anti-Lyt 2.1 and anti-Lyt 2.2 antibodies. The potential of using immobilized antibodies for determining allotypes of HLA antigens and the proportions of leukocyte subsets was discussed, but there has been little subsequent development of this procedure. We tested this method for the binding of human Raji (B-cell Burkitt lymphoma) or CCRF-CEM (T-cell ALL) cells to antibodies against CD3, CD4, CD8, and CD19 as dots on a glass slide with inconsistent results. Therefore, we developed the antibody microarray, called the LD Array (in memory of Lee Dixon), described here.

## MATERIALS AND METHODS

**Cell Lines.** CCRF-CEM (acute lymphoblastic leukemia), Raji (Burkitt lymphoma), and HL-60 (acute promyelocytic leukemia) were obtained from the American Type Culture Collection (Manassas, VA) and were grown in RPMI 1640 medium (Trace Scientific, Melbourne, Victoria, Australia) supplemented with 10% fetal bovine serum (CSL Limited, Parkville, Victoria, Australia) and 50 µg/ml gentamicin sulfate (Life Technologies, Inc., Grand Island, NY).

**Construction of Antibody Microarrays.** The procedure has been described in a PCT application (16). A PixSys 3200 Aspirate and Dispense System (Cartesian Technologies, Irvine, CA) was used to construct a rectangular array (0.72 cm × 0.4 cm) of 60 different 5-nl antibody dots air-dried on a film of nitrocellulose bound to a glass microscope slide (Fast Slides; manufactured by Grace Biolabs, Bend, OR; supplied by Schleicher and Schuell, Keene, NH). Antibodies were purchased from the following companies: Coulter and Immunotech from Beckman Coulter (Gladesville, NSW, Australia), PharMingen from BD Biosciences (North Ryde, NSW, Australia), and Biosource International from Monarch Medical (Stafford City, Queensland, Australia). They were reconstituted as recommended, frozen in aliquots at -20°C with 0.1% (w/v) BSA (Sigma-Aldrich, Castle Hill, NSW, Australia), and used at concentrations ranging from 25 to 1000 µg/ml, as supplied for FACS analysis. After application, antibody dots were visualized by eye on a white light box for quality control, and the corners of the array were marked with a pencil. The nitrocellulose was then blocked with 5% (w/v) skim milk (Diploma; Bonlac Foods, Melbourne, Victoria, Australia) in PBS (90 min at room temperature), washed with water, dried, and stored at 4°C with desiccant. Each batch of slides was tested with cell lines and/or frozen peripheral blood leukocytes or leukemia cells of known phenotype to check antibody-binding activities.

**Binding of Leukocytes to the LD Array.** Blood was drawn from normal subjects and leukemia patients for this project with informed consent and approval of the Human Ethics Committee of the University of Sydney (reference number 99/07/07). Leukocytes were isolated from peripheral blood (treat-

ed with EDTA or heparin to prevent clotting) using Histopaque (Sigma-Aldrich), washed in PBS, resuspended in PBS containing 1 mM EDTA to a density of 10<sup>7</sup> cells/ml, and incubated with the LD Array for 30 min at room temperature (100 µl of suspension/slide), and unbound cells were then gently washed off with PBS. Inclusion of EDTA significantly reduced nonspecific attachment of cells to the blocked nitrocellulose. Heat-inactivated human AB serum (10% (v/v); Sigma-Aldrich) was added to AML cells that otherwise attached to all of the antibody dots because of presumed Fc receptor binding. Arrays were then fixed for at least 1 h in PBS containing 1% (v/v) formaldehyde (Sigma-Aldrich), 2% (w/v) glucose, and 0.05% (w/v) sodium azide and washed in PBS.

**Data Recording and Analysis.** Bound leukocytes were observed wet by nonconventional dark-field microscopy using an Olympus BX60 fluorescence microscope (Olympus, North Ryde, NSW, Australia) with a UPlan 4X objective. The condenser was set at the phase 1 position, and a green filter was placed over the light source. Images were recorded and analyzed using a SenSys digital cooled CCD camera (1317 × 1035 pixels; Photometrics, Tucson, Arizona), PCI Frame Grabber, and "V" version 3.5 for Windows image processing and analysis software (Digital Optics, Auckland, New Zealand). Images were processed using Adobe Photoshop version 5.0 software, and dot densities were scored by eye by comparison with a set of standard dots of increasing intensity. This semiquantitative method of scoring compared favorably with quantification using ImageQuant version 3.3 software (Molecular Dynamics, Sunnyvale, CA). Even after drying, fixed arrays could be observed microscopically if moistened with PBS.

**Flow Cytometry.** Isolated peripheral blood leukocytes (10<sup>6</sup> cells) were incubated for 15 min at room temperature with FITC- or PE-conjugated antibodies (Coulter or Immunotech; concentrations as recommended by manufacturers) and 2% (v/v) heat-inactivated human AB serum (Sigma-Aldrich). After washing in FACS buffer [PBS with 0.1% (w/v) BSA and 0.1% (w/v) sodium azide], cells were resuspended in fixative and analyzed on a FACScalibur flow cytometer, with a 488-nm air-cooled argon-ion laser, running CELLQuest Software (Becton Dickinson, San Jose, CA).

**Confocal Microscopy.** After incubation with leukocytes, LD Arrays were fixed and incubated with a 1:1 mixture of CD3-FITC (Coulter; diluted 1 in 200 with FACS buffer) and CD19-PE (Immunotech; undiluted) for 10 min at room temperature. Specificity of staining was checked using FITC- and PE-conjugated isotype control antibodies. Slides were washed in PBS and mounted using a SlowFade Antifade Kit (Molecular Probes; from Bioscientific, Gympie, Australia). Slides were examined with a Leica TCS NT Confocal System (Microsystems, Heidelberg, Germany), using a Plan Apo 100×/1.40-0.7 oil immersion objective. Images were processed using Adobe Photoshop version 5.0 software.

## RESULTS

Preliminary experiments showed that unwanted cellular aggregation and nonspecific binding of leukocytes to the nitrocellulose could be minimized by using the following conditions: washing of cells to remove exogenous nutrients; addition of a chelating agent (EDTA); and reducing incubation temperature from 37°C to 22°C (data not shown). Under these conditions, little or no cell aggregation was detected microscopically on the antibody dots, and background binding was absent or minimal.

Fig. 1 illustrates the distinct binding patterns of three well-characterized cell lines incubated with an LD Array of 60 antibodies and observed by nonconventional dark-field microscopy. The intensities of the dots reflect the densities of the cells bound to the antibodies at the locations shown in the key (Fig. 1a). CCRF-CEM cell binding was high on antibody dots CD4, CD5, CD7, CD8, CD38, CD44, CD45, CD45RO, CD71, and CD95 and low on CD3 and CD52 (Fig. 1b). Raji cell binding was high on CD10, CD19, CD20, CD21, CD22, CD23, CD37, CD38, CD45, CD45RA, CD52, CD71, CD79b, CD80, and CD95; moderate on sIg; and low on CD154, κ (Fig. 1c). HL-60 binding was high on CD4, CD13, CD33, CD44, CD64, CD71, and CD117; moderate on CD11b, CD11c, CD15, CD38, CD45, CD45RO, CD95, and KOR (CD66c); and low on CD8, CD14, and CD16 (Fig.

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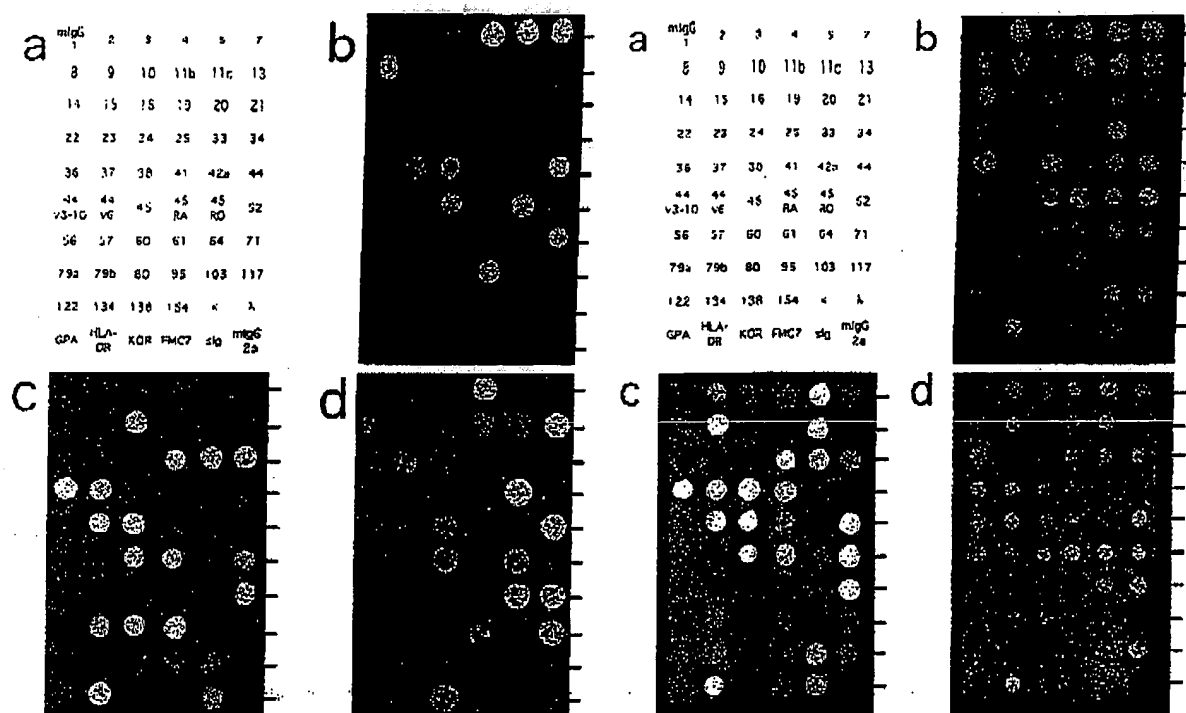


Fig. 1. Binding patterns of human cell lines using the LD Array. *a*, key to antibody identification. *b*, CCRF-CEM; *c*, Raji; *d*, HL-60. The numbers in the antibody key refer to antibodies against the corresponding CD antigens; mlgG1 and mlgG2a are murine isotype control antibodies; 44 v3-10 and 44 v6 are antibodies against CD44 variants 3-10 and 6;  $\kappa$ ,  $\lambda$ , GPA, HLA-DR, KOR, FMC7, and slg are antibodies against human  $\kappa$  and  $\lambda$  light chains, GPA (CD235a), HLA-DR, KOR-SA3544 antigen (CD66c), FMC-7, and surface immunoglobulin.

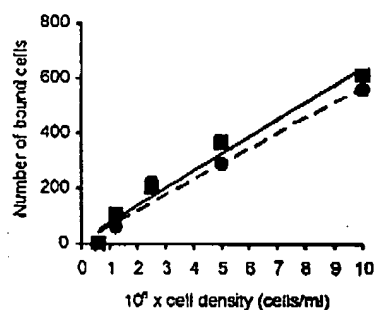


Fig. 2. Relationship between cell density of the sample and number of cells binding to antibody dots on LD Arrays. Serial 2-fold dilutions of a suspension of Raji cells ( $10^7$  cells/ml) were tested on LD Arrays. The number of cells bound to antibody dots for HLA-DR (■) and CD38 (●) are plotted against the density of cell suspensions. Regression analysis of data for HLA-DR (—) and CD38 (—) gave coefficients of correlation ( $R^2$ ) of 0.97 and 0.96, respectively.

1e). Fig. 2 shows the near linear relationship between cell density of the sample and number of bound cells on an antibody dot when Raji cells at densities up to  $10^7$  cells/ml were bound to antibody dots HLA-DR and CD38. Little or no binding was observed at a sample density of  $6 \times 10^7$  cells/ml.

Fig. 3 shows binding patterns of leukocytes from a normal subject (Fig. 3h) and a variety of leukemia patients. Normal peripheral blood leukocytes (Fig. 3h) were captured by more than 30 of the antibody dots. Antibodies against T-cell antigens (CD2, CD3, CD4, CD5, and

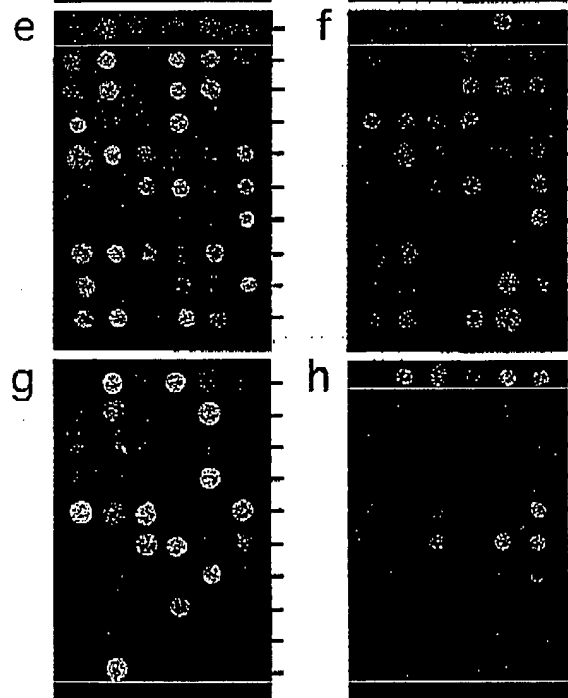


Fig. 3. Binding patterns of human leukocytes using the LD Array. The leukocyte abbreviation, source, and cell density of the sample appear in brackets. *a*, key to antibody identification; *b*, normal peripheral blood leukocytes (subject 1;  $4 \times 10^6$  cells/liter); *c*, CLL (patient 1;  $30 \times 10^6$  cells/liter); *d*, CLL (patient 2;  $9 \times 10^6$  cells/liter); *e*, HCL (patient 3;  $16 \times 10^6$  cells/liter); *f*, mantle cell lymphoma (patient 4;  $19 \times 10^6$  cells/liter); *g*, AML (patient 5;  $190 \times 10^6$  cells/liter); *h*, T-cell ALL (patient 6;  $17 \times 10^6$  cells/liter). The numbers in the antibody key refer to antibodies against the corresponding CD antigens as defined in the legend to Fig. 1.

## ANTIBODY MICROARRAY FOR LEUKEMIA DIAGNOSIS

CD7) bound more cells than those for B-cell antigens (CD19, CD20, CD21, CD22, CD23, and CD24), reflecting the 70:30 ratio of T- to B-lymphocytes, which collectively comprise 20–40% of leukocytes in peripheral blood (17). Antibodies against CD44 and CD52 bound leukocytes at high density, consistent with expression of these antigens on a wide range of leukocytes. Intermediate levels of leukocytes bound to CD13 and CD33 dots, reflecting monocyte/granulocyte concentrations in peripheral blood (collectively >40% of leukocytes; Ref. 17). Antibodies against CD41 and CD42a bound platelets, whereas anti-glycophorin A (CD235a) bound erythrocytes. Both types of cells are found in fresh leukocyte preparations.

Samples from CLL patients with high leukocyte counts ( $>30 \times 10^9$  cells/liter) showed fewer positive dots ( $<25$ ; Fig. 3c), and these were of uniform density, reflecting the predominance of monoclonal CLL cells over normal leukocytes ( $4-10 \times 10^9$  cells/liter; Ref. 17). For CLL patients with lower leukocyte counts ( $8-14 \times 10^9$  cells/liter; Fig. 3d), the pattern of binding was still distinguishable from normal leukocytes because dots corresponding to B-cell antigens were of higher or equivalent density to T-cell dots. We have not tested CLL patients with leukocyte counts of less than  $8 \times 10^9$  cells/liter, but the CLL pattern was still evident when CLL cells and normal leukocytes were mixed in a 1:3 ratio (equivalent to a total blood leukocyte count of  $5 \times 10^9$  cells/liter), indicating that the LD Array could be used to detect earlier stages of B-cell leukemias or lymphomas (data not shown).

Leukocytes from a HCL patient (Fig. 3e) were distinguished from CLL by strong binding to antibodies against CD103 and FMC-7 and an absence of CD23 and CD5, consistent with the typical HCL immunophenotype. The small number of cells bound to the anti-CD5 dot were T-cells, identified by confocal microscopy of cells bound to this dot that were fluorescently labeled with soluble anti-CD3 and anti-CD20 (data not shown). The NHL (Fig. 3f) had a B-cell immunophenotype distinguishable from both CLL and HCL (CD5<sup>+</sup>/CD79b<sup>+</sup>/CD103<sup>+</sup>/FMC-7<sup>+</sup>). The pattern of leukocytes from an AML patient was biphenotypic (Fig. 3f), with expression of the T-lymphocyte marker CD2 in addition to antigens of the monocytic lineage (CD4, CD11b, CD33, CD36, CD38, CD64, and HLA-DR). This immunophenotype was confirmed by flow cytometry (data not shown). ALL leukocytes had a T-cell immunophenotype (Fig. 3h). The immunophenotypes for HCL, NHL, and T-ALL (Fig. 3, e, f, and h) obtained with the LD Array correlated well with flow cytometric data supplied by pathology laboratories (15–21 antigens; data not shown).

Determination of the expression of 48 antigens on leukocytes from two CLL patients using the LD Array correlated closely with flow cytometry (Table 1), particularly for antigens expressed at high levels.

A high level of binding occurred when FACS analysis revealed antigen expression on 75–100% of the total population (CD5, CD19, CD20, CD24, CD37, CD44, CD45RA, CD52, and HLA-DR; patient 7). Moderate binding correlated with antigen expression on 35–75% of the population (CD9, CD11b, and CD21; patient 7), whereas low binding occurred when 15–35% of cells expressed antigen (CD22, CD45RA, CD79a, and GPA; patient 8). Binding was generally negative or +/- when 0–15% of cells were positive by FACS. In a few cases, cell binding was lower than expected from FACS results (CD11c, CD79a, CD95, and CD154 for patient 7), usually reflecting low expression of these antigens. However, low antigen expression did not always correlate with poor binding, suggesting that some antibodies (CD22, CD23, and CD71 for patient 8) bound cells more strongly than others, although the strongly binding antibodies were used at lower concentrations (25 or 50  $\mu\text{g}/\text{ml}$ ) than the others (200  $\mu\text{g}/\text{ml}$ ). Some dots showed +/- binding of cells, whereas FACS results were negative (CD2, CD7, CD9, CD103, CD117, and CD122

Table 1. Comparison of LD Array and flow cytometric analyses for two CLL samples

Dot array results are expressed as level of cell binding to each antibody dot: - (none), +/- (very low), + (low), ++ (moderate), +++ (high), ++++ (very high). Flow cytometric analysis results are expressed as percentage of cells showing fluorescence for each antigen. The +/- to +++ results shown in brackets denote intensity of fluorescence compared to cells with control antibody (FITC- or PE-conjugated murine IgG1).

Surface antigens	CLL (patient 7)		CLL (patient 8)	
	Dot array	FACS (%)	Dot array	FACS (%)
2	-	2	+/-	7
3	-	3	-	5
4	-	4	-	0
5	+++	99 (-)	+++	90 (+)
7	+/+	7 (-)	+/+	0
8	-	0	-	0
9	+/+	75 (-)	+/+	0
10	-	0	-	0
11b	+/+	57 (-)	-	0
11c	+	44 (-)	+/+	3 (++++)
13	-	0	-	0
14	-	2	+/+	10 (+/-)
15	-	0	-	0
16	-	0	-	0
19	+++	98 (++)	+++	94 (+)
20	+++	99 (++)	+++	85 (++)
21	+/+	70 (-)	+	22 (+/-)
22	+/+	75 (-)	+/+	70 (+/-)
23	+/+	13 (-)	+/+	68 (+/-)
24	+++	98 (++)	+++	94 (++++)
25	+/+	68 (-)	+/+	11 (+/-)
33	-	0	-	0
34	-	0	-	0
36	-	0	+/+	9 (++)
37	+/+	90 (++)	+/+	97 (++)
38	-	0	+/+	97 (++)
41	-	0	+/+	11 (+/-)
42a	-	0	+/+	0
44	+/+	99 (++)	+/+	100 (++)
45	+/+	100 (++)	+/+	100 (++)
45RA	+/+	93 (-)	+	26 (++)
45RO	+/+	22 (+/-)	+/+	86 (++)
52	+/+	100 (++)	+/+	100 (++)
56	-	0	-	0
57	-	0	-	8 (+/-)
60	-	0	-	7 (+/-)
61	-	0	-	11 (+/-)
71	+/+	75 (-)	+/+	61 (+/-)
79a	+/+	35 (+/-)	+	15 (++)
95	-	13 (+/-)	-	14 (+/-)
103	-	0	+/+	7 (+/-)
117	-	0	+/+	0
122	-	0	+/+	0
154	-	23 (+/-)	+/+	0
GPA	-	0	+	17 (+)
HLA-DR	+/+	99 (++)	+/+	98 (++)
KOR	-	4	-	0
FMC7	+/+	35 (+/-)	+/+	13 (+/-)

for patient 8), suggesting the detection of minor subpopulations of cells by dot array ( $10^6$  cells/array) but not by FACS (5000 cells counted). However, these results should be interpreted with caution, because a +/- score represents  $<50$  cells/antibody dot and may not be significant.

Analysis of frozen and freshly isolated normal leukocytes or leukemia cells gave comparable dot patterns using the LD Array. However, some antibodies were occasionally negative for frozen cells (CD9, CD11b, CD13, CD14, CD15, CD16, CD56, CD57, CD79a, CD95, and CD154), suggesting either low binding affinity or loss of these surface antigens because of shedding or epitope destruction. Even if some antigens were missed on frozen cells analyzed by the LD Array, the extensive panel of antibodies permits some variation in the dot patterns between samples without changing a diagnosis derived from the consensus pattern.

Leukocyte samples from 20 CLL patients showed predictably and distinctly different dot patterns from samples from 20 normal subjects, as summarized in Fig. 4. Highly significant differences ( $P < 0.0005$ )

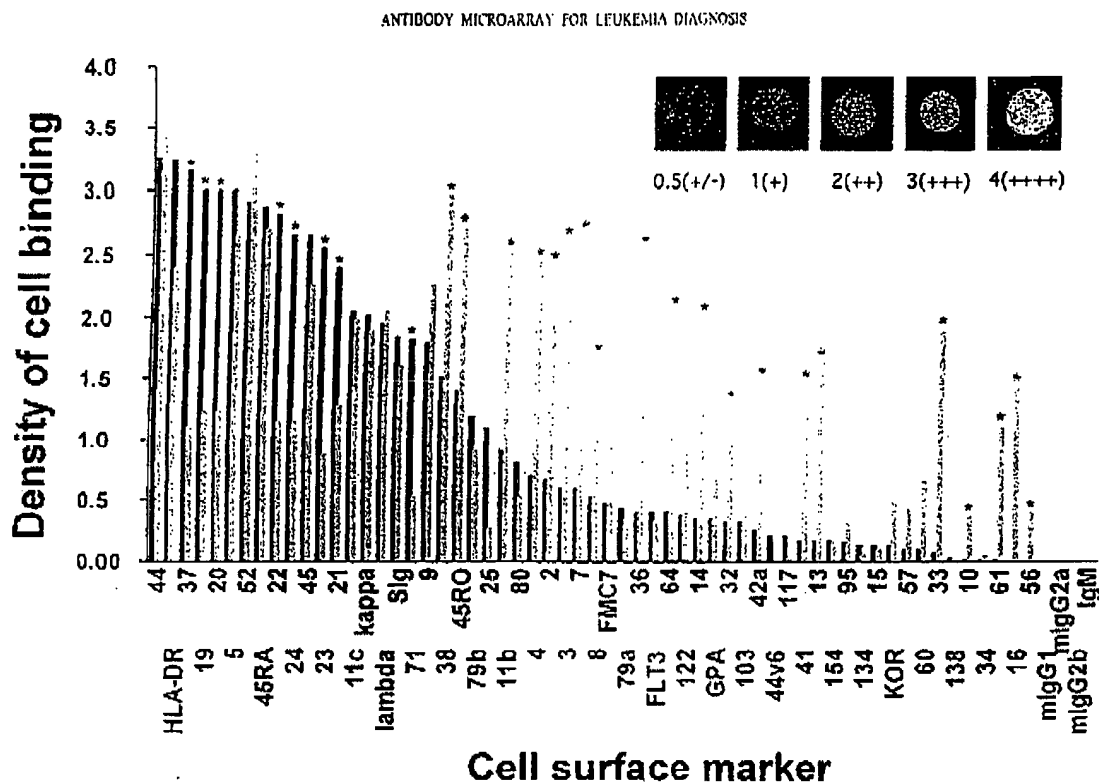


Fig. 4. Immunophenotypes of normal peripheral blood leukocytes and CLL cells. Leukocytes from peripheral blood of 20 normal individuals and 20 CLL patients (with leukocyte counts ranging from  $8 \times 10^9/\text{liter}$  to  $250 \times 10^9/\text{liter}$ ) were tested using the LD Array, and the density of cell binding was scored by comparison with a set of standard dots of increasing intensity, as shown in the inset. The average values for CLL cells were calculated for each antibody and are plotted in descending order (black bars). The average values for normal peripheral blood leukocytes are also shown (grey bars). Statistical significance ( $P < 0.0005$ ) of differences between CLL and normal peripheral blood leukocytes was determined by two-sample Student's *t* test assuming equal variances and is shown by an asterisk (\*).

between normal and CLL leukocytes occurred for 28 antigens, marked with an asterisk. The consensus pattern of antigen expression for CLL leukocytes in descending order of cells bound was: CD44, HLA-DR, CD37, CD19, CD20, CD5, CD52, CD45RA, CD22, CD24, CD45, CD23, CD21, CD11c, and CD71.  $\kappa$  and  $\lambda$  light chain expression defined leukemia monoclonality. The antigens providing the best discrimination between CLL and normal peripheral blood leukocytes were CD19, CD20, CD21, CD22, CD23, CD24, and CD37. In addition, CLL samples showed a significant reduction in binding to antibodies specific for T cells (CD2, CD3, CD4, and CD7) and various myeloid cells (CD4, CD11b, CD13, CD14, CD16, CD32, CD33, CD36, CD41, CD42a, CD61, and CD64), reflecting the high ratio of leukemia B cells:normal leukocytes. In agreement with the literature, some, but not all, of the CLL samples were positive for cell surface markers such as CD9, CD11b, CD11c, CD25, CD38, CD45RO, CD71, CD79a, CD79b, CD80, CD122, FMC-7, and FLT3 (CD135). Two samples were negative for CD23. Normal peripheral blood leukocytes showed relatively high levels of platelets, which were easily recognizable microscopically on antibodies to CD9, CD36, CD41, CD42a, CD60, and CD61 (Fig. 3b). Samples from leukemia patients generally showed significantly reduced levels of platelet binding, reflecting the relatively low proportion of this cell type in the blood (Fig. 3, c-h). In some samples, GPA dots revealed RBC contamination (Fig. 3, e and f). It was subsequently shown that removal of RBCs from leukocyte preparations by osmotic lysis (lysis buffer, 0.15 M ammonium chloride/10 mM potassium hydrogen carbonate/0.1 mM EDTA) did not significantly alter the leukocyte binding pattern.

Leukocytes from CLL samples bound at high densities to anti-CD5 (Fig. 3, e and d), consistent with the known expression of CD5 on CLL cells. The density of binding was high compared with the binding of CLL cells to antibodies against other T-cell markers (e.g., CD7) but equivalent to binding to B-cell markers (e.g., CD20). As shown in Fig. 5, cell populations bound to an antibody dot could be further characterized by labeling with soluble anti-CD3-FITC (green) and anti-CD19-PE (red) and observing by confocal microscopy. Normal peripheral blood leukocytes binding to CD5 and CD7 dots were clearly T cells (Fig. 5, a and b), whereas those binding to CD20 were B cells (Fig. 5c). In contrast, CLL cells bound to CD5 were predominantly B cells (Fig. 5d), and relatively low numbers of T cells were observed on CD5 and CD7 (Fig. 5, d and e). CD20 bound B cells exclusively from both samples (Fig. 5, e and f), with a predominance of leukemic B cells in the blood of the CLL patient.

## DISCUSSION

The LD Array would enable rapid screening of many leukocyte samples for a large range of surface antigens, particularly if the recording and analysis of dot patterns were automated. Analyses of hematological malignancies using the LD Array could potentially identify not only the lineage and maturation stage of the cells but also new prognostic markers. Uncommon surface antigens could be identified for subsequent detection of minimal residual disease after chemotherapy and as targets for immunotherapy with humanized monoclonal antibodies [18, 19].

## ANTIBODY MICROARRAY FOR LEUKEMIA DIAGNOSIS

## NORMAL

## CLL

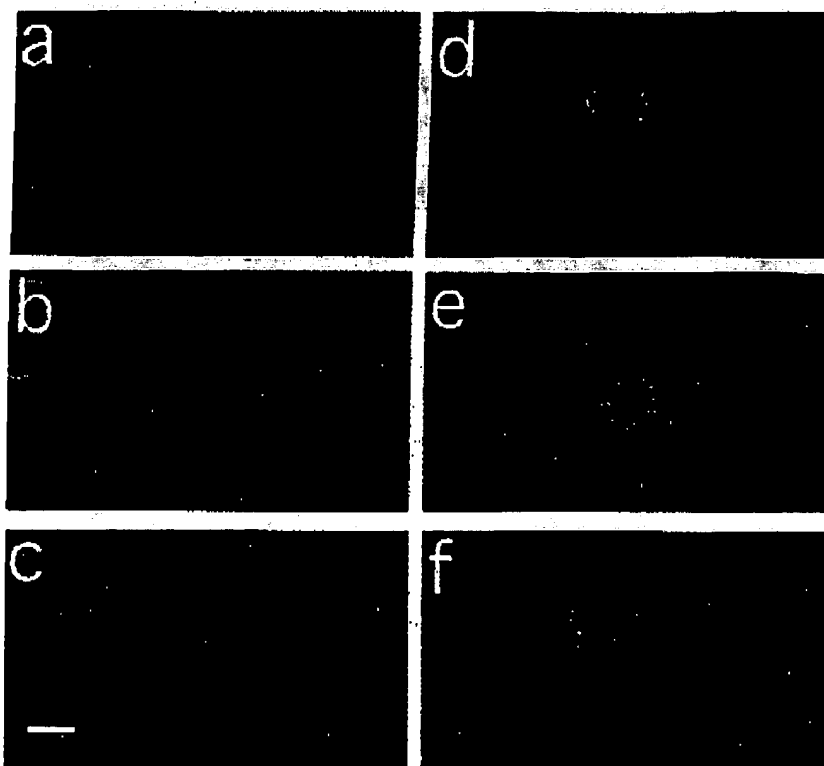


Fig. 5. Confocal microscopy. Detection of CD3 (green fluorescence) and CD19 (red fluorescence) on leukocytes from (a, b, c) a normal individual and (d, e, f) a CLL patient (patient 9;  $13 \times 10^9$  cells/liter), bound to three different antibodies on an LD Array: a and d, anti-CD3; b and e, anti-CD7; c and f, anti-CD20. White scale bar, 10  $\mu$ m. The binding of fluorescently labeled CD antibodies to cells captured on an LD Array is described in "Materials and Methods."

Although the LD Array is a powerful tool, it would not provide all of the information obtained by FACS analysis, e.g., multiparameter analysis on single cells or level of antigen expression/cell. Although the LD Array allows semiquantitative determination of relative densities of subpopulations of cells of distinct immunophenotypes, absolute quantification may not be possible. At equilibrium, the number of cells captured by an antibody dot depends not only on cell numbers but also on the affinity of interaction, concentration of antibody in the dot, level of expression of the antigen on the cell surface, and its steric accessibility to the antibody immobilized on nitrocellulose. Computerized quantification of cell density (pixel intensity) on dots depends not only on cell number but also on cell size and morphology. The main strength of the LD Array is speed and extensive immunophenotyping, enabling pattern recognition using large arrays of microscopic antibody dots. LD Array slides can be stored over desiccant at 4°C for prolonged periods (>6 months) without significant loss of binding activity, although some of these antibodies are not stable in aqueous solution at 4°C over this period. The translucent nature of moistened nitrocellulose permits microscopic examination of bound cells. If recognition of consensus dot patterns from low-density leukemias proves to be problematic against the background heterogeneity of peripheral blood leukocytes, subpopulations of cells on any antibody dot can be observed by fluorescence microscopy (Fig. 5).

The LD Array is now being used to establish consensus patterns of antigen expression for diagnosis of leukemias other than CLL. Accumulation of LD Array results for large numbers of leukemias will provide a database enabling diagnosis of blood-borne cancers by

pattern recognition. A relational database has been described for the diagnosis of hematological malignancies using immunophenotyping by flow cytometry (20). Automatic processing of slides, recording of dot patterns, and computerized quantification and pattern recognition are currently under development. Standardization of these processes will be required to enable the direct comparison of data sets from different laboratories. Additional work is required to determine the optimum concentration of each antibody. The current method uses antibodies supplied at concentrations appropriate for use in FACS analysis. It may be possible to further enhance the sensitivity of the LD Array by the selection of different antibody concentrations and/or hybridoma clones.

CLL is either stable or progressive (21, 22). Recent studies correlate stable disease with a low bcl-2/bax ratio (23) and progressive disease with high serum levels of CD23 and interleukin-8 (24–26). However, attempts to correlate CLL immunophenotype with prognosis have yielded inconclusive results (27). The ability of the LD Array to screen large numbers of CLL samples for expression of a wide range of CD antigens may lead to the recognition of new CLL subgroups. In addition, the testing of frozen leukemia samples may allow a retrospective correlation between immunophenotype and disease progression. At the recent Seventh Workshop and Conference on Human Leukocyte Differentiation Antigens (Harrogate, United Kingdom), 81 new CD antigens were defined, the last being CD247. Monoclonal antibodies against these additional CD antigens will soon be available commercially, further extending the scope for analysis of leukocyte populations using the LD Array.

## ANTIBODY MICROARRAY FOR LEUKEMIA DIAGNOSIS

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